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(54) Title: DETECTION OF A CHARACTERISTIC ANTIGEN FROM CARCINOMA IN BODY FLUIDS

(57) Abstract

A method for diagnosing the presence of carcinoma in a patient and for evaluating the effectiveness of a carcinoma treatment regime on a given patient is disclosed. The method involves extracting a body fluid from the patient to be diagnosed and isolating from the body fluid an extracellular protein portion of a growth receptor protein which growth receptor is known to be associated with the particular carcinoma selected. The isolated protein portion is treated in a manner so as to render the portion antigenic after which it is brought into contact with antibodies which are immunospecific for the treated protein portion. Binding reactions between the antigens and antibodies are observed and compared with the standard to thereby diagnose the presence of a carcinoma and/or evaluate the effectiveness of a treatment regime. Specific methods are disclosed.

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DETECTION OF A CHARACTERISTIC ANTIGEN FROM CARCINOMA IN BODY FLUIDS

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Technical Field

The invention relates to the field of diagnostic technology. More particularly, it concerns the detection of excessive amounts of growth factor receptor proteins in human body fluids such as sera in order to diagnose (1) the presence of a tumor; (2) the projected growth rate of a tumor; or (3) the effectiveness of a method of treating the carcinoma associated with the receptor proteins.

Background Art

The association of characteristic antigens with various tumors is well known. The antigens may be associated with the surface of the tumor and/or may be secreted into the plasma or other body fluids. Indeed,

some such tumor antigens are used as indices of the presence of tumors. Perhaps the best known of these is carcinoembryonic antigen (CEA) which is found in cancers of the gastrointestinal tract, particularly the colon. It has been detected in the sera of patients with neoplastic as well as nonmalignant diseases. Another antigen, alpha-feto protein (AFP) has been detected in the serum of adults with hepatomas and testicular blastomas. Although these antigens do not raise autochthonous antibodies, they can be detected in the sera using antibodies raised in allogeneic or xenogeneic hosts. (Stites, D.P., et al., Basic and Clinical Immunol, 5th ed., (1984), Lange Medical Publications, Los Altos, California, page 226).

The HER-2/neu oncogene, which is also referred to as c-erb-2, is a member of the erbB-like oncogene family, and is related to, but distinct from, the epidermal growth factor (EGF) receptor. The HER-2/neu gene has been shown to be amplified in human breast cancer lines. More specifically, HER-2/neu was found to be amplified from 2- to greater than 20-fold in 30% of the tumors as compared to normal tissue. Slamon et al., Science 235:177-182 (9 Jan 1987). This suggests the overexpression of this gene may be involved in human breast cancer. Hynes et al., Journal of Cellular Biochemistry 30:167-173 (1989).

The cellular gene encoding the receptor for epidermal growth factor (EGF) has considerable homology to the oncogene of avian erythroblastosis virus. In a human mammary carcinoma, the DNA sequence has been identified as being related to V-erbB. King et al., Science 229:974-978 (6 Sept 1985).

Amplification of the erbB/EGF receptor and structurally-related gene HER-2 (erbB-2), have previously

been detected in a variety of human tumors. Krause et al., EMBO Journal Vol. 6, pp. 605-610 (1987).

The nucleotide sequence of EGF receptor and HER-2/neu cDNA and a predicted amino acid sequence are given in Ullrich et al. Nature 309:418-425 (1984) incorporated herein by reference to disclose such sequences.

The above-referred to publications are merely a small sample of the available publications relating to carcinomas and their detection and/or the relationships between carcinomas and the increased amounts of certain proteins. Although vast amounts of research has been done in these areas, present technologies for diagnosing for the presence of carcinomas generally involve the surgical removal of the tissue suspected of being carcinogenic and subsequent analysis of that tissue. The use of surgery is not only inconvenient and expensive but presents substantial risks to the patient. Further, once a tumor has been surgically removed and the patient has been treated in an attempt to prevent regrowth of the tumor, it is generally not possible to use surgery in order to determine the effectiveness of the treatment in preventing tumor regrowth. The present invention provides a system wherein antibody antigen conjugates are formed and determinations are made based on the formation of these conjugates with respect to the presence of tumors, the growth rate of tumors and the effectiveness of therapies in reducing tumor growth.

It has not been possible to use serological antibody tests to detect all tumor markers for which diagnosis is desired. In particular, the product of the proto-oncogene $p185^{HER-2}$, the HER-2 protein, is known to be associated with breast carcinoma. Although this marker can be detected on the surface of tumor cells, it has not been possible to assay for the presence of this

protein in body fluids such as sera. Accordingly, diagnosis, absent recovery of the intact tumor, has not been possible. The present invention, by permitting analysis of sera for the presence of marker proteins for specific receptors, permits ready diagnosis even prior to surgery.

Summary of the Invention

The present invention provides a method for detecting (by the use of antibodies) the extracellular portion of receptor proteins which are related to particular carcinomas. In particular, the invention provides antibodies which are both sensitive and selective with respect to detecting portions of epidermal growth factor (EGF) receptor proteins and portions of HER-2/neu receptor proteins and other extracellular proteins known in the art as proto-oncogene receptors. By detecting the presence of one of these proteins within human body fluids such as sera and determining the relative amounts of these proteins with respect to normal patients without carcinomas, it is possible to determine (1) the presence of carcinomas; (2) the potential growth rate of the carcinomas; and (3) the recurrence of carcinomas surgically removed and/or chemically treated.

The present invention provides a method for determining which patients have carcinomas and for determining the prognosis of patients which have been determined to have carcinomas. Methods of the invention are carried out by detecting the extracellular portion of receptor proteins which are shed from carcinoma cells. The detection is carried out by extracting and then treating a biological fluid into which the extracellular proteins are shed. The fluid is preferably human blood serum obtained from a patient either suspected of having a carcinoma or which is known to have that carcinoma and

which is being diagnosed with respect to the effectiveness of treatment of that carcinoma. The fluid is treated in a manner which allows the extracellular proteins being detected to become immunogenic with respect to previously generated antibodies. The treated fluid is then contacted with the antibodies which are immunospecific towards the extracellular region of the proteins being detected. A variety of different detection means known to those skilled in the art can be utilized in order to determine the presence or absence of a protein/antibody conjugate.

The relative amount of protein detected vis-a-vis the amount detected in a healthy patient makes it possible to diagnose the presence of a carcinoma and the potential growth rate of the carcinoma. By determining the amount of a particular extracellular protein in a patient at a given time and comparing that amount with an amount detected at a different time, it is possible to determine the potential effectiveness of treatments being utilized to eliminate the carcinoma.

One aspect of the invention is directed to a method to diagnose a patient for the presence or absence of a human breast tumor characterized by the HER-2 marker protein. The method employs serological techniques using antibodies directed to the extracellular region of the HER-2 marker. By proper treatment of the serum or other fluid before serological testing, strong and detectable reaction between the antibody and the tumor marker is ensured.

In accordance with this aspect of the invention, it is possible to detect the presence or absence of HER-2 protein in a biological fluid by treating the fluid with a reagent which effects the permanent lysis of disulfide bonds (of the extracellular portion of HER-2 protein), such as the combination of a

reducing agent and an alkylating agent, and contacting the resulting treated fluid with an antibody specific for the extracellular region of the HER-2 protein, followed by detecting the specific reaction, if any, of the antibody with a component of the fluid. In other aspects, the invention is directed to antibodies specific for this and other extracellular portions of receptor protein and to reagent kits suitable for conducting the method of the invention.

Another aspect of the invention relates to an assay for the detection of the extracellular domain of the proto-oncogene epidermal growth factor (EGF) receptor which is released from human prostate carcinoma cells. In accordance with the method, extracellular bodily fluids such as prosthetic fluid, serum or urine are treated using highly specific and selective immunological reagents for proteins that contain the amino acid sequence from the extracellular domain of the EGF receptor. In that the extracellular domain of the EGF receptor is directly involved in the growth of human carcinomas the level of expression of this protein is indicative of the growth rate of the cells. Accordingly, abnormally high concentrations of the protein are indicative of the presence of a tumor, and specifically a prostate tumor. By determining the amount of the protein present in a bodily fluid and comparing that amount with a pre-established normal, tumors can be detected without the use of surgery or biopsies. Further, by comparing the concentration of such proteins in a given patient with the concentration of the proteins at a later time during tumor treatment the efficacy of any treatment protocol can be readily evaluated with respect to the specific patient being treated.

An important object of the present invention is to provide a method for detecting the relative amount of

particular extracellular receptor proteins in human body fluids such as sera with respect to the amount present in the same body fluid of healthy patients so as to determine the presence of and projected growth rate of carcinomas.

Another important object of the present invention is to provide a method for determining the relative efficacy of carcinoma therapeutic treatments by determining the relative amounts of and differences in the amounts of extracellular receptor proteins relative to the amounts of such proteins present in healthy patients and/or determining the differences in the amounts of such proteins present in the same patient at different times.

Another object of this invention is to provide a method to diagnose the presence or absence of a condition characterized by the shedding of the extracellular portion of particular receptor proteins.

Another object of the present invention is to provide diagnostic methodology which comprises extracting a biological fluid from a human, treating the fluid in such a manner so as to render the extra cellular proteins therein antigenic and immunogenic, contacting those proteins with previously-generated antibodies which specifically bind to such treated proteins and comparing the amount of antibody antigen reactions with the amount of reactions occurring in a normal patient and thereby determining the likelihood of a carcinoma in the patient whose body fluids were tested.

Another object of the invention is to provide such a method of diagnosis which involves treating a biological fluid into which the extracellular portion of HER-2 protein is shed with a reagent for reducing and alkylating and effecting the permanent lysis of the disulfide bonds of the protein and then contacting the

lysed protein with an antibody which is immunospecific for some portion of the extracellular HER-2 protein and detecting the presence of a reaction with the antibody.

An important feature of the present invention is that it provides a method for determining the relative amount of particular extracellular proteins with respect to the amount of these proteins present in healthy patients and/or present in a single patient at different times.

Another feature of the present invention is that the biological fluids being tested for the presence of the extracellular proteins must be treated in order to render the extracellular proteins immunospecific with respect to the antibodies used for their detection.

An important advantage of the present invention is that it provides a method for evaluating the efficacy of various carcinoma therapeutic treatments by providing information with respect to changes in the amount of extracellular protein present in the sera during the period of treatment.

Another advantage of the present invention is that the presence of breast and/or prostate carcinomas can be determined utilizing human serum and without the use of surgically removed tissue.

Yet another advantage of the present invention is that the assay is both sensitive and specific and can be carried out efficiently and economically.

Another advantage of the present invention is that body fluids in addition to sera such as urine and prostatic fluid can be extracted and assayed in accordance with the assay process of the invention.

Another feature of the invention is that the extracellular fragments which are detected in the body fluids are derived from and reflect the amount of cellular forms of proto-oncogene receptors and the amount

of such receptors can be compared with normal patients and thereby correlated with the likelihood of carcinomas.

Another advantage of the present invention is that it allows for a determination of the amount of proto-oncogene receptors the amounts of which are related to aggressive tumor growth which information can be related to the survival rates of patients and/or the effectiveness of treatments offered to particular patients.

These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the assay method and reagents as more fully set forth below, reference being made to the accompanying figure forming a part hereof.

Brief Description of the Drawings

The invention may be better understood and its numerous objects, features and advantages will become more apparent to those skilled in the art by reference to the accompanying figures as follows:

Figure 1 shows a Western blot of cellular extracts and media from human breast carcinoma cells using specific HER-2 antibodies of the invention;

Figure 2 is an immunoblot of cell extracts and conditioned media from human breast carcinoma cells with antipeptide antibody to a site in the extracellular domain of the HER-2 protein;

Figure 3 is an immunoprecipitation of (^{35}S)methionine labeled 110K sEGFR from extracellular media and 170K EGFR from cell extracts of A431 cells; and

Figure 4 shows the detection of 125 kd protein from the extracellular medium of human prostate carcinoma cells using an antibody generated against the extracellular domain of the human EGF receptor.

Modes of Carrying Out the Invention

Before the present method, assay systems and reagents are described, it is to be understood that this invention is not limited to the particular methods, assays, reagents or antibodies described as such may, of course, vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

It should be noted that as used in this specification and the appended claims, the singular form "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus for example, reference to "a reducing agent" includes mixtures of reducing agents, reference to "an antibody" includes one or more antibodies of the type herein described, which are immunospecific for the desired protein and reference to "the method of detection" includes one or more methods which might be utilized by those skilled in the art having read the present disclosure.

Abnormally high amounts of growth factor receptors encoded by proto-oncogenes (e.g., the proto-oncogene epidermal growth factor receptor) are linked to the maintenance and progression of human cancers. The receptors which have been found to be amplified most often in connection with human carcinomas are the epidermal growth factor (EGF) receptor and the HER-2/neu receptor. Both of these receptors have been found in several types of human carcinomas in increased amounts as compared with the amounts of these receptors present in normal tissue. Enhanced amounts of the HER-2 receptors are particularly prevalent in oncogenic tissue found in breast epithelial cells. Further, breast tumors with

elevated amounts of HER-2 receptors, as compared with other breast tumors, have more aggressive growth, and patients with tumors having these elevated amounts of HER-2 receptors show decreased survival times, as compared with patients having tumors with lower levels of such receptors.

Previously, the relationship between the over-expression of EGF receptors and/or HER-2 receptors and malignant growth was determined by surgically removing the tissue suspected of being malignant and analyzing for the presence of such receptors. However, since the surgical removal of such tissues for testing is not only expensive and inconvenient but presents considerable risks to the patient, there remained a need to detect the presence of such receptors by nonsurgical means. The present invention provides such nonsurgical means for detecting the presence of such receptors and determining the amount of such receptors present relative to normal patients and/or relative amounts found in a body fluid of a given patient at different points in time. By making a determination of the relative amount of such receptors, diagnosis can be made with respect to the presence of or projected growth rate of malignant tumors and/or the efficacy of a given treatment designed to retard or eliminate such malignant growth.

The present invention provides methodologies which are used in combination with specific antibodies which are immunospecific for detecting extracellular fragments of proteins from EGF receptors and HER-2 receptors. The methodology of the present invention utilizes a body fluid such as sera obtained from a patient, which sera will contain extracellular protein fragments of the receptors to be detected. The sera are treated in a specific manner and then exposed to the antibodies which have been developed in accordance with

the present invention. The binding of the immunoreactive segments of the receptors to the antibodies is then detected. A determination is then made with respect to the amount of extracellular protein which is circulating in the patient's sera. The amount is compared with a healthy patient and/or the same patient whose sera were extracted at a different point in time during the treatment. By comparing the relative amounts of antigen-antibody conjugates formed, a determination can be made with respect to whether the patient has a carcinoma and/or whether the patient's treatment regime is effectively retarding the growth of the carcinoma. The amount of extracellular protein detected in a given body fluid will vary depending upon the body fluid extracted and may vary based on factors such as the size, sex and condition of the patient. Accordingly, in order to obtain reliable results, the assayed body fluid should be compared with a healthy patient of the same size, age and sex of the patient being tested. Although several types of body fluids can be used to carry out the assays of the invention, it is most convenient to rely on the sera due to the consistent presence of such extracellular protein in sera, and the consistency of the amount of such a protein in patients based on the presence or lack of presence of a particular carcinoma.

It should be pointed out that when the assay methodology of the present invention is applied in order to detect the amount of extracellular HER-2 released from normal cultured breast epithelial cells no such extracellular protein is detected due to the extremely low level of the protein being generated. Thus, it can be deduced that particular assays can be developed whereby the extracellular proteins in normal patients will not be detected due to the lack of sensitivity of the assay. Thus, normal patients will show no

extracellular proteins of the type being tested for in the assay, whereas patients who have developed carcinomas will show varying levels of such proteins depending upon the size and growth rate of the carcinoma. When assaying the sera of a patient known to have a carcinoma which patient is being treated, the sera should be extracted and assayed on a regular basis, preferably every seven to fourteen days. By taking regular assays and determining the change in the amount of extracellular protein, the effectiveness of a treatment regime can be readily determined.

Methods of detecting extracellular portions of receptor proteins associated with cell growth (e.g., HER-2)

In general, the methodologies of the present invention can be applied to predict the growth rates of various types of tumors in that the invention relates the relative amount of extracellular proteins (found in sera and associated with tumors) to the amount of such extracellular proteins found in a normal patient. However, EGF receptor amplification occurs in the carcinomas of several tissue types, while enhanced HER-2 expression may be particular to breast epithelial cells. Further, since different proteins are involved with respect to EGF and HER-2 receptors, different antibodies must be developed in order to obtain both a sensitive and specific immunospecific assay. It should be noted that some of the methodology described below with respect to developing antibodies and assay methodologies for HER-2 proteins may, in certain circumstances, be applicable to developing antibodies and assays to be used in connection with other proto-oncogene growth factor receptors and putative growth factor receptors. Examples of such receptors include colony stimulating factor receptors,

EGF receptor proteins, platelet derived growth factor receptor, erbB-3, and C-kit.

In accordance with this aspect of the invention, a biological sample, such as serum, is treated with a reagent to provide a form of the HER-2 which is immunoreactive with antibodies specific for the extracellular region of this marker. When treating HER-2 proteins of a particular type described herein, preferred reagents are combinations of a reducing agent to effect cleavage and an alkylating agent to stabilize the resulting sulfhydryls. However, when different extracellular protein are being treated, the treatment should be designed so as to render the protein as antigenic as possible so that it will readily bind to the immunospecific antibodies.

As used herein, "reducing agent" refers to an agent of reducing power sufficient to result in the cleavage of disulfide bonds (of the extracellular portion of HER-2 protein) so that proteins subjected to the reducing agent which contain cysteyl residues contain these as cysteine, not involved in disulfide linkages. Suitable reducing agents are well known in the art, and include mercaptoethanol, dithiothreitol, and the like.

"Alkylating agents" are those which stabilize the sulfhydryl groups derived from the reduction. These, too, are well known in the art, but include, for example, iodoacetate which results in the formation of an S-carboxymethyl derivative, iodoacetamide, and ethylene-amine.

In some instances, the stable or "permanent" disruption of disulfide bonds can also be accomplished by alternative methods such as oxidation to yield cysteic acid residues. However, the combination of reduction and alkylation is preferred. It is necessary that the disulfide bonds of the extracellular portion of the HER-2

protein remain disrupted for a sufficient period of time to allow for the assay to be conducted. This requires that the antibody bind to the disrupted protein and that this antibody protein conjugate be detected.

In the method of the invention, a biological fluid, typically a serum, is first treated with a reagent, as stated above, to disrupt any disulfide linkages. The preferred approach is reduction followed by alkylation. The treated fluid, then, contains the secreted HER-2 protein in a form which does not contain disulfide bonds. When the treated fluid is then contacted with a suitable amount of antibodies raised against the extracellular region of the HER-2 protein, preferably the 15 amino acid sequence characterizing this region, the antibodies are capable of reacting with the HER-2 marker.

The reaction of the antibodies is detected by a variety of means known to practitioners in the art. The detection may be, for example, using Western blot techniques, or the antibodies may be conjugated to a solid support and the binding of antigens detected by a second antibody in a sandwich-type assay. Detection reagents include suitable labels such as radioactive, fluorescent, or chromogenic labels, or enzymes which can then be detected in the presence of substrate solution. All of these detection techniques are well known, and a multiplicity of ELISA and RIA assays can be designed using the treated biological fluid and the antibodies of the invention.

By making use of known detection agents as indicated above, those skilled in the art can develop standards by which a normal reaction in terms of a normal amount of extracellular proteins can be judged. By comparing the normal reactions standard against the reaction obtained from testing a sample from any

particular patient the likelihood of that patient having a carcinoma can be determined in accordance with a preferred embodiment of the invention and an assay kit is provided wherein a range of color-coded standards are provided wherein each standard has been associated with a normal patient and a patient with high amounts of extracellular protein to the extent that such is associated with a carcinoma in that patient.

Those skilled in the art will recognize that it is possible to carry out the methodologies of the present invention by utilizing different antibodies developed with respect to different amino acid sequences taken from EGF and HER-2 extracellular proteins portions. Once a particular portion of the extracellular protein of either of these receptor proteins is obtained, attempts can be made to generate specific antibodies to that portion of the protein by means well known to those skilled in the art. If immunospecific antibodies can be generated, then the general methodologies of the invention can be carried out. More specifically, if antibodies can be generated which are immunospecific to a particular portion of the extracellular portion of either of these receptor proteins, then those antibodies can be used to determine the relative amount of the extracellular protein present within the patient's serum relative to the amount of such extracellular protein present in a normal patient. Further, such antibodies can be used to determine the relative amount of such extracellular proteins present in the patient's serum at different points in time in order to make evaluations with respect to tumor growth rate and/or the effectiveness of a given treatment regime. Although a variety of different sequences from the extracellular portion of these receptor proteins might be used and a variety of different antibodies might be generated, the following is a description of a particular

amino acid sequence which has been found to be useful in connection with developing antibodies which are specific for detecting the extracellular portion of HER-2 receptor proteins present within human serum.

The amino acid sequence of the extracellular portion of HER-2 protein is known and is incorporated herein by reference. (See Coussens et al., "Tyrosine Kinase Receptor...," Science, 230:1132-1139 (Dec. 1985) incorporated herein by reference). However, others have not made use of the protein or portions thereof to develop sensitive and specific assays to detect the presence of the protein with an antipeptide antibody. One embodiment of the present invention allows for such an assay by using a 15 amino acid sequence as follows: GLY_{151} GLY_{152} VAL_{153} LEU_{154} ILE_{155} GLN_{156} ARG_{157} ASN_{158} PRO_{159} GLN_{160} LEU_{161} CYS_{162} TYR_{163} GLN_{164} ASP_{165}. However, it is pointed out that others skilled in the art will (using this disclosure) be able to obtain other sub-sequences from the HER-2 protein and treat such sequence as needed (such as by reducing and alkylating to disrupt sulfide bonds) to obtain a sequence which will be specific to the extracellular portion of the HER-2 protein and bind to an antipeptide antibody thus making other assay systems possible.

As used herein, "antibodies specific for the extracellular portion of the HER-2 protein" refers to antibodies which react with this region with much higher affinity than that with which they are capable of binding either any other proteins or the intracellular regions of HER-2. Typically, these antibodies are raised by injection of a suitable mammalian subject, such as rabbits or mice, with the extracellular fragments, preferably the 15 amino acid sequence characterizing this portion of the protein. Immunization protocols are optimized using standard methods and titers are

determined by standard ELISA or RIA methods using the immunogen as antigen in the assay. When sufficient titers are raised, the polyclonal antisera may be used in the methods of the invention, or spleen cells or PBLs may be immortalized and used for the production of monoclonal forms of antibodies which specifically react with the extracellular portion of HER-2.

As will be recognized by those skilled in the art, it is possible to generate antibodies in accordance with recently developed procedures such as disclosed by E. Harlow and D. Lane, Antibodies: A Laboratory Manual, Coldspring Harbor Laboratory (1988). In situations where antipeptide antibodies are used, either in a western (immuno) blots, immunoprecipitation, or in ELISA, excess synthetic peptide can be added to the test sample and should block the reaction provided the reaction is specific. This makes it possible to test for the specificity of the antigen antibody reaction.

Any biological fluid may be used in the assay, but it is understood that blood serum is preferably used in the diagnosis of human patients. It is expected that the levels of secreted HER-2 marker will be more readily detected in this fluid than, for example, in urine.

In another embodiment of the present invention, prostrate carcinomas can be detected by the presence of extracellular receptors associated with such carcinomas. When attempting to detect extracellular proteins associated with prostate carcinomas, it is often desirable to utilize a prostate fluid. When attempting to detect carcinomas associated with the bladder, it is desirable to use urine as the source material for the body fluid being assayed. However, depending on the type of carcinoma and/or receptors which are being detected, some adjustments may be made with respect to the body fluid which is to be used for carrying out the assay.

Those skilled in the art can determine those fluids which contain higher percentages of such extracellular proteins by using assay methodologies as disclosed herein. The location of the carcinoma as well as the type of carcinoma must be taken into consideration when determining the type of body fluid to be used in the assays in order to make the most effective use of the detection systems of the invention. However, by adjusting the sensitivity of the assays, different body fluid can be used without substantially affecting the results obtained by the present invention.

The following example is given so as to provide those of ordinary skill in the art with a complete disclosure and description of how to carry out the assay method of the invention and is not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to insure accuracy with respect to numbers used (e.g., amounts, molecular weights, etc.) but some experimental errors and deviation should be accounted for. Unless indicated otherwise, the temperature is room temperature and pressure is at or near atmospheric.

General means of generating antibodies

It is pointed out that the present invention can be utilized in connection various methodologies for generating antibodies. Specifically, the present invention utilizes segments of the extracellular portions of either EGF or HER-2 proteins which can generate an immune response. The immune response must be specific or the readings obtained will be meaningless. In order to develop antibodies which can be used in connection with the present invention, it is often necessary to modify in some manner the portions of the extracellular portions of

these protein receptors so that they are both immunogenic and capable of generating specific immune responses.

One means for modifying such proteins so that they are antigenic and specifically bind to amino specific antibodies has been discussed above wherein the disulfide bonds of the peptides are broken. However, it is pointed out that there are a variety of different techniques for modifying proteins so that such proteins are antigenic and bind into amino specific antibodies. The modification of such proteins and the ability to generate amino specific antibodies is described in detail in E. Harlow and D. Lane, Antibodies: A Laboratory Manual, Coldspring Harbor Laboratory (1988) which is incorporated herein by reference.

Another possible means of modifying such protein segments is disclosed within "Protein Structure and Design," UCLA Symposium on Molecular and Cell Biology, New Series, Vol. 69, Dale Oxender (ed.), Alan R. Liss, Inc., New York, 1987. See, specifically, "The Chemical Synthesis of Structured Peptides Using Covalent Hydrogen-Bond Mimics," by T. Arrhenius et al. In accordance with the method disclosed by Arrhenius et al., covalent mimics of the hydrogen bonds within peptide structures were developed in order to obtain the particular protein folds present within natural proteins. By using this methodology, very small segments of larger proteins could be placed in a form where they are immunogenic and capable of generating a very specific immune response.

When generating antibodies it is desirable to use particularly preferred sources of extracellular proteins and such can be obtained from a tissue culture media. By obtaining the proteins from the tissue culture media, the consistency of the protein can be more accurately controlled. It is also pointed out that it is

at times desirable to use synthetic proteins which contain a specific sequence known to be associated with extracellular proteins. When developing assays to be used in connection with the detection of breast carcinoma, amino acid sequences obtained from epidermal growth factor receptor proteins are generally used. The epidermal growth factor receptor cDNA nucleotide sequence and a predicted amino acid sequence is disclosed within A. Ullrich et al. Nature Volume 307 pp. 418-425 (May 31, 1984) which article is incorporated herein by reference for its disclosure of the epidermal growth factor receptor nucleotide sequence and predicted amino acid sequence. By using the amino acid sequence disclosed by Horowitz et al., those skilled in the art will be able to determine sequences which would be particularly useful with respect to their ability to generate antibodies and which sequences would be uniquely associated with the receptor protein which is to be detected. Thus, by correctly choosing the particular sequences, the assay can be made both highly selective and sensitive.

Production of antibody to the N-terminus of $p185^{HER-2}$

Antibodies were prepared by immunizing rabbits with a synthetic peptide of 15 amino acids corresponding to the sequence from residues 151 -165 (Gly-Gly-Val-Leu-Ile-Gln-Arg-Asn-Pro-Gln-Leu-Cys-Tyr-Gln-Asp) at the N-terminus of $p185^{HER-2}$ (Coussens, L., Yang-Feng, T.L., Liao, Y.C., Chen, E., Gray, A., McGrath, J., Seeburg, Ph.H., Libermann, T.A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. (1985) Science 230, 1132-1139) incorporated herein by reference. The peptide was synthesized by the solid phase peptide synthesis method using 9-fluorenylmethoxycarbonyl (Fmoc) polyamide continuous flow technique essentially as described

(Dryland, A., and Sheppard, R.C. (1986) J Chem Soc Perkin Trans I:125-132) incorporated herein by reference. Fmoc amino acid esters and Pepsyn KA resin were obtained from MilliGen, division of Millipore. The completed peptide was cleaved from the resin and were assessed for purity by HPLC using a C18 column (Waters, division of Millipore). Peptide, purified by HPLC, was subjected to amino acid analysis. One milligram of peptide was mixed with 3 mg of hemocyanin in 1 ml of 0.5% 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC) for 30 min at room temperature. About 100 μg of peptide conjugated to hemocyanin was emulsified in an equal volume of Freund's complete adjuvant and injected subcutaneously into rabbits. Booster injections using 100 μg of conjugated peptide in incomplete Freund's adjuvant were given every two weeks following the initial injection. Antibody production was monitored by immunoblotting cell extracts from BT474 cells. Antibodies specific to the C-terminus of $p185^{HER-2}$ were prepared as described (Lin, Y.J., Li, S., and Clinton, G.m. (1990) Mol. Cell. Endoc. 69:111-119) incorporated herein by reference.

Choosing the amino acid sequences from the kinase domain for synthesis of the peptide antigen

When selecting the amino acid sequence from the catalytic domain to which the antipeptide antibody will be directed, it is important to consider whether the epitopes will be collinear with the primary sequence. In the absence of information on the three dimensional structure of the catalytic domain, whether the sequence is conserved may be considered. Patterns of amino acid sequence conservation can most easily be assessed by consulting the publication of S.K. Hanks, A.M. Quinn and T. Hunter, Science 241:42 (1988), incorporated herein by reference, in which these authors aligned the sequences

within the catalytic domains of 65 different protein kinases. Attention should be directed not only to the eleven conserved subdomains (numbered I through XI) but also to the patterns of conservation within and outside of these subdomains. For example, within subdomain VI, the sequence HRDLRAAN (HRD) for the Src family (residues 384-391) is one of the most highly conserved segments in the tyrosine kinase family and may, therefore, represent a site that is structurally or functionally important. Other considerations when choosing the amino acid sequence, include the length and the chemical properties of the amino acid side chains. See R. Gherzi et al., J Bio Chem, 264:8627 (1987); M. Makowske et al., J Bio Chem, 264:16,155 (1989) and R.A. Lerner, Nature, 299:596 (1982), each of which is incorporated herein by reference.

Conjugation of the synthetic peptide

There are several procedures for conjugating the peptide to a carrier protein. We have found that crosslinking synthetic peptides with carbodiimide by the following procedure has yielded antibodies which are reactive with highly conserved sequences in the interior of the EGFR.

1. Dissolve 2.5 mg of the synthetic peptide in 1 ml water. Determine absorbance at 214 nM or at the wavelength of maximum absorbance for the particular peptide.
2. Add 7.5 mg hemocyanin (thyroglobulin works also) in 100 µl of water containing 0.5 mg/ml crosslinker 1 ethyl-(3-dimethylaminopropyl)-carbodiimide HCl (EDC) (Sigma). Vortex and let stand at room temperature for 30 min.

3. Dilute to 3 ml with water and put into dialysis tubing with a pore size that will allow free peptide to diffuse out. Dialyze against 100 ml of water overnight.

4. Determine absorbance of dialysate and calculate the amount of peptide in the dialysate to determine the efficiency of conjugation. Alternatively, spot an aliquot of dialysate onto paper and react with ninhydrin to estimate amount of free peptide. We have found that 40 to 50% of the peptide is usually conjugated.

We have also assessed the immunogenicity of peptide conjugated to hemocyanin using glutaraldehyde as the crosslinking reagent. Glutaraldehyde efficiently conjugated the peptide to the carrier protein and the conjugated peptide generated antibody reactive to the immobilized peptide in an ELISA. However, we have found that these antibodies did not immunoprecipitate the EGFR if the cognate sequence was in the interior of the protein. The conformation of the peptide that is crosslinked with glutaraldehyde may be different than the conformation of the same sequence when it is located at an internal site in the protein. Antipeptide antibodies to sites in the flexible C-terminal domain, on the other hand, bound to the EGFR whether glutaraldehyde or carbodiimide was used to prepare the immunogen.

Another procedure evaluated for producing immunogen is the synthesis of a heptalysine core to which peptide monomers were attached according to the method of D.N. Posnett, H, McGrath and J.P. Tam, J. Biol. Chem 263:1719 (1988) and J.P. Tam, Proc. Natl. Acad. Sci. 85:5409 (1988), incorporated herein by reference. Peptides attached to the polylysine core have been found

to be immunogenic, thereby eliminating the need for a carrier protein. However, because the synthesis of the peptide on the polylysine core was more difficult and there did not appear to be improvement in the titer or in the proportion of immunized rabbits that produced antibodies, there seems little reason to use this procedure.

Example 1
Detection of HER-2

Two human breast carcinoma cell lines, BT474 and SK-BR-3, were used. An extract from BT474 and media from both cell lines were concentrated, reduced and alkylated. The reduced, alkylated samples were electrophoresed in a polyacrylamide gel, and the proteins transferred onto nitrocellulose.

The nitrocellulose blot was reacted with antibody prepared to the characteristic 15 amino acid segment in the extracellular region of the HER-2 protein using standard techniques to provide a Western blot. The results are shown in Figure 1.

In Figure 1, lane A, is a cellular extract of BT-474. This shows that the antibody reacts with a variety of proteins in the extract; the darkest band, at about 200 kd, is the complete HER-2 protein, which is internal to the cell.

Lanes B and C are obtained from media of BT-474 and of SK-BR-3 respectively. These results show that a major protein of 130 kd is shed which reacts with antibody specific to HER-2 protein. This protein is released from these two breast carcinoma cell lines, but not from normal breast cells. The identity of the 130 kd protein as the extracellular region of the HER-2 protein

has been established by independent techniques. It is pointed out that the molecular weight of the protein may vary depending on the method employed and the source of the protein.

The present inventors point out that the amount of extracellular protein released from epithelial cells derived from normal breast tissues are at least 50 to 100-fold less than the amount of extracellular protein released from breast carcinoma cells. Due to the extremely high differential with respect to the amount of extracellular protein which will be present when a carcinoma is present, the assays of the present invention provide an assay which is sensitive even when taking into account a substantial margin of error. Due to the substantial difference in the amount of extracellular proteins which will be present in the sera of a normal patient as compared to a patient with a breast carcinoma, assays of the invention can be readily devised so that a healthy patient without a carcinoma will show no reaction whatsoever (i.e. show no extracellular proteins) whereas a patient with a breast carcinoma will show a strong color-coded reaction on the assay.

Example 2

Detection of extracellular forms of EGF

As indicated above, various methodologies, reagents and materials used in connection with obtaining a particular segment of one type of extracellular receptor protein and generating antibodies to that can be used in connection with a different segment of the same extracellular protein or a segment from a different extracellular protein. In order to detect extracellular forms of EGF receptor, antibodies were generated to a region of the EGF receptor that extends outside the cells. Methods of generating such antibodies are known

to those skilled in the art. Utilizing such antibodies, measurements were made of a 110K soluble form of the EGF receptor that is secreted into the extracellular media of the A431 human carcinoma cells (see Figure 2). With respect to the 110K soluble form of the EGF receptor, reference is made to Weber et al. (1984), Science, 224:294-297, and to Basu et al. (1989), Molecular and Cell Biology, 9:671-677.

By utilizing the 110K soluble form of EGF receptor protein (EGFR), it was possible to prepare antibodies which are sensitive, specific, and which can be used for the detection of extracellular forms of EGFR that are released from carcinomas and present within human sera.

Referring specifically to Figure 2, which shows an immunoblot of immunoprecipitation of (^{35}S)-methionine-labeled 110K sEGFR from extracellular media, and 170K EGFR from cell extracts of A431 cells. In Lane A there is present 110K protein from the extracellular media immunoprecipitated by antibodies to the EGF receptor. Lane B shows (^{35}S)-methionine-labeled media immunoprecipitated with control, preimmune sera. Lane C contains 170K EGFR immunoprecipitated from (^{35}S)-methionine-labeled cell extracts from A431 cells.

Example 3

Prostate Cancer Detection Assay

The general procedures of the present invention as described above can be followed in order to detect extracellular proteins associated with different types of malignant growth. An advantage of the present invention is that it is possible for different types of carcinoma to exhibit the same type of extracellular protein associated with the abnormal growth. Accordingly, once a particular type of antibody has been generated with

respect to a particular type of extracellular protein that antibody may well be useful with respect to the detection of different types of carcinomas. A demonstration of the ability of the methodology of the present invention to detect human prostate carcinoma based on the extracellular protein domain of the EGF receptor is described here.

The human prostate carcinoma cell line BT474 was incubated with ^{35}S-methionine/cysteine for 8 hrs. The isotopically labeled proteins were then chased with excess cold methionine/cysteine for 0 hrs (lanes a,d) 12 hrs (lanes b,e) and 24 hrs (lanes c,f). The extracellular medium (lanes a,b,c) and the cell extract (lanes d,e,f) were immunoprecipitated with antisera against the extracellular domain of the EGF receptor and the immune complexes were analyzed by SDS-gel electrophoresis and autoradiography. The results are shown in Figure 4. The carrot in the left margin of Figure 4 marks the 125 kD extracellular EGF receptor. The carrot on the right margin of Figure 4 marks the cellular 170 kD EFG receptor.

The present assay invention and assay detection kit has been shown and described herein in what is considered by the inventors to be the most practical, and preferred embodiments. It is recognized, however, that departures may be made therefrom which are within the scope of the invention, and that obvious modifications will occur to one skilled in the art upon reading this disclosure.

CLAIMS

What is claimed is:

1. A method of determining the effectiveness of a cancer therapy being applied to a patient suffering from a cancer, comprising the steps of:

(a) extracting a biological fluid sample from the patient, which fluid will contain extracellular portions of a receptor protein known to be associated with the cancer of the type the patient is suffering from;

(b) treating the biological fluid so as to generate immunospecific antigens from the extracellular portions of the receptor proteins;

(c) contacting the antigens with antibodies which are immunospecific for the antigens;

(d) determining the amount of receptor protein present within a given unit of biological fluid;

(e) repeating steps (a)-(d) with respect to a second biological fluid sample obtained from the same patient at a different time from when the first biological fluid sample was extracted; and

(f) calculating the change in the amount of extracellular protein present in a unit amount of the patient's biological fluid and thereby determining the effectiveness of the cancer therapy.

2. The method as claimed in claim 1, wherein the first and second extracting of biological fluid are at points in time which are at least one day apart.

3. The method as claimed in claim 2, wherein the periods between extracting the first sample and extracting the second sample is at least seven days.

4. A method for determining the rate of growth of a tumor within a human patient, comprising the steps of:

extracting a first blood serum sample from a patient;

treating the blood serum in a manner so as to generate immunospecific antigens from extracellular portions of receptor proteins present within the blood serum;

extracting a second blood serum sample from the same patient at a different point in time from when the first sample was extracted;

treating the second sample of blood serum in the same manner as the first sample in order to generate antigens from extracellular portions of receptor proteins present in the blood serum;

separately contacting antigens from the first sample and second sample with antibodies specific to such antigens; and

determining the relative difference in the amount of antigen-antibody conjugates formed using the first sample and the second sample, and thereby determining the rate of growth of the tumor in the patient.

5. A method to diagnose the presence or absence of a condition characterized by the shedding of the extracellular portion of HER-2 protein which method comprises:

treating a biological fluid into which said protein is shed with a reagent which effects the permanent lysis of disulfide bonds;

contacting the resultant with an antibody which is immunospecific for the extracellular region of HER-2 protein; and

detecting the presence or absence of the reaction of said antibody with a component of the biological fluid.

6. The method of claim 5 wherein the biological fluid is serum.

7. The method of claim 5 wherein the antibody is immunospecific for the 15 amino sequence characteristic of the extracellular protein.

8. The method of claim 5 wherein the reagent is the combination of a reducing and an alkylating agent.

9. The method of claim 8 wherein the reducing agent is mercaptoethanol and the alkylating reagent is iodoacetate.

10. The method of claim 5 wherein the condition is human breast carcinoma.

11. The method as claimed in claim 5 wherein the antibody is immunospecific for the 15 amino acid sequence as follows: GLY_1 GLY_2 VAL_3 LEU_4 ILE_5 GLN_6 ARG_7 ASN_8 PRO_9 GLN_{10} LEU_{11} CYS_{12} TYR_{13} GLN_{14} ASP_{15}.

12. An isolated portion of the HER-2 protein which has been treated in such a fashion so as to be immunoreactive with an antipeptide antibody, wherein the portion of the protein is specific to the extracellular portion of HER-2 protein.

13. The isolated portion of the HER-2 protein as claimed in claim 12 wherein the protein has the sequence as follows: GLY_1 GLY_2 VAL_3 LEU_4 ILE_5 GLN_6 ARG_7 ASN_8 PRO_9 GLN_{10} LEU_{11} CYS_{12}-TYR_{13} GLN_{14} ASP_{15}.

14. A monoclonal antibody preparation which is immunoreactive with the 15 amino acid antigenic region characteristic of HER-2 protein, but does not substantially react with other antigens, wherein the 15 amino acids are:
GLY_1 GLY_2 VAL_3 LEU_4 ILE_5 GLN_6 ARG_7 ASN_8 PRO_9 GLN_{10} LEU_{11} CYS_{12} TYR_{13} GLN_{14} ASP_{15}.

15. A diagnostic kit for the detection of the presence or absence of the extracellular portion of HER-2 protein in a biological fluid, which kit comprises

antibodies specifically immunoreactive with said extracellular region of HER-2 protein; and

reagents for the detection of immunoreaction of said protein with the antibodies.

16. A method of diagnosing the presence of a condition characterized by the shedding of an extracellular portion of a growth receptor protein known to be associated with abnormal cancerous growth, which method comprises:

treating a biological fluid into which said portion of said protein is shed in such a manner so as to render the portion of the protein antigenic;

contacting the treated protein portion with an antibody which is amino specific for the treated protein portion;

detecting the presence of a binding reaction between the antibody and the treated protein portion;

comparing the amount of binding reactions between the antibody and the treated protein portion with a known standard and thereby diagnosing the presence or absence of a carcinoma.

FIG. 1

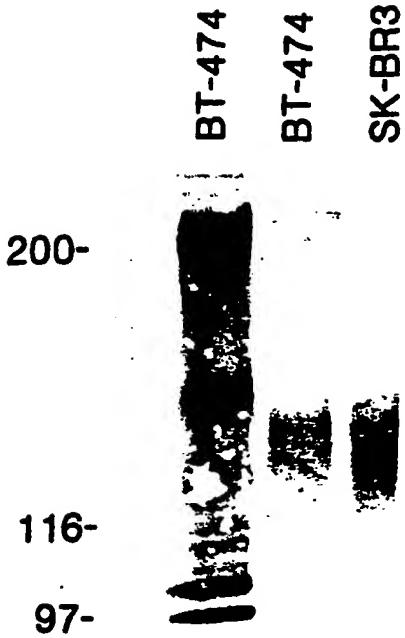
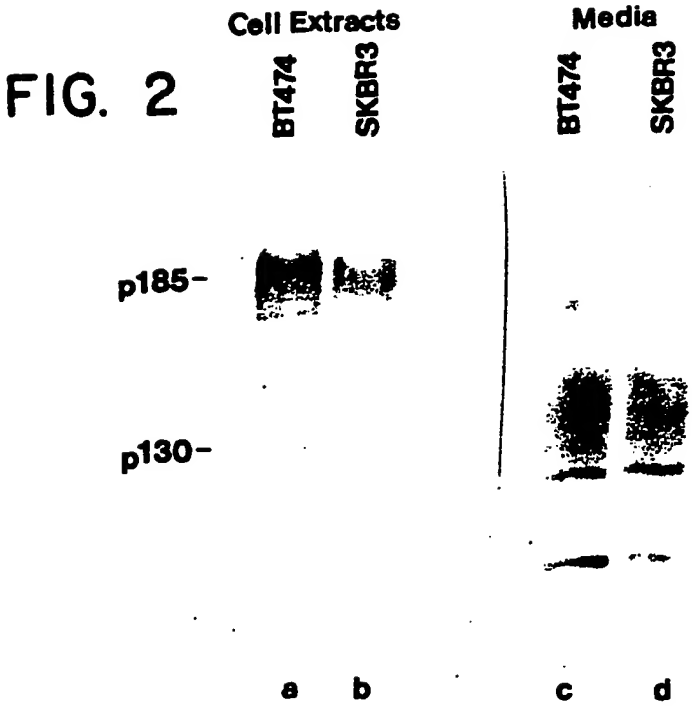

BT-474
BT-474
SK-BR3
200-
116-
97-
A
B
C

FIG. 2


Cell Extracts
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a
b
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d

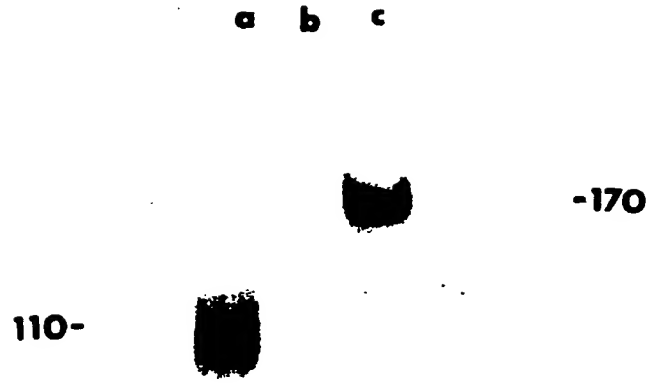

FIG. 3

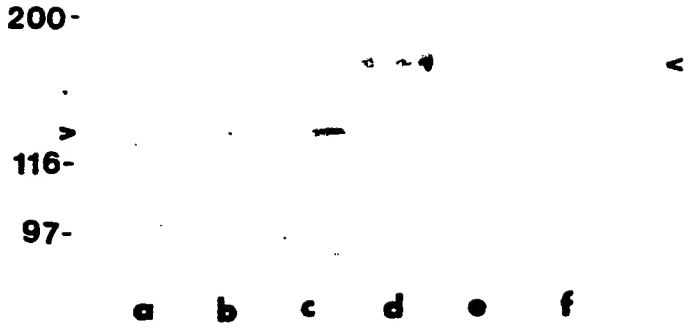

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/00561

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

[3] According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): G01N 33/53, 33/536, 33/577; C07K 15/00

U.S.Cl.: 435/7.1, 7.23; 436/501, 536, 548; 530/350

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/7.1, 7.23, 7.9, 967; 436/501, 536, 547, 548, 813; 530/350, 828

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [5]

DIALOG SEARCH

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [15]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	The EMBO Journal, vol. 6, no. 3, issued 1987, Kraus et al., "Over-expression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms", pp. 605-610. See entire Abstract and page 609, column 1, lines 28-44 and entire column 2.	1-3
Y	Biological Abstract, No: 88110012 Zeillinger et al., "HER-2 Amplification Steroid Receptors and Epidermal Growth Factor Receptor in Primary Breast Cancer, ONCOGENE, 4(1): 109-114, 1989. See entire abstract.	1-3
Y,P	Biological Abstract, No: 89125097, Lin et al., "Insulin and Epidermal Growth Factor Stimulate Phosphorylation of P185H-E-R-2 in the Breast Carcinoma Cell Line BT474," see abstract MOLCELL ENDOCRINOL, 69(2-3): 111-120, 1990.	1-3

[15] Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
30 April 1991	22 MAY 1991
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Florina B. Hoffer

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y,P	Biological Abstract, No: 89094902, Bacus et al., "HER-2-NEU Oncogene Expression and DNA Ploidy Analysis in Breast Cancer, see abstract, ARCH PATHOL LAB MED 114(2): 164-169, 1990.	1-3
Y,P	Biological Abstract, No: 90065113, Berchuck et al., "Overexpression of HER-2-NEW is Associated with Poor Survival in Advanced Epithelial Ovarian Cancer", CANCER RES, 50 (13): 4087-4091, 1990. See abstract.	1-3
Y,P	Biological Abstract, No: 90041436, Tandon et al., "Association of the 323-A3 Surface Glycoprotein with Tumor Characteristics and Behavior in Human Breast Cancer", CANCER RES, 50 (11): 3317-3321, 1990. See entire abstract.	1-3
Y,P	Biological Abstract, No: 90064611, Read et al., "Hormonal Modulation of HER-2-NEU Protooncogene Messenger RNA and P185 Protein Expression in Human Breast Cancer Cell Lines", CANCER RES, 50 (13): 3947-3951, 1990. See abstract.	1-3
Y	US,A, 4,803,169 (LINSLEY ET AL) 07 February 1989, See entire Abstract, and column 2, lines 1-43.	1-3
Y,P	US,A, 4,921,790 (O'BRIEN) 01 May May 1990. See entire Abstract.	1-3
Y,P	US,A, 4,946,774 (OH) 07 August 1990. See entire Abstract.	1-3
Y,P	US,A, 4,954,436 (FROEHNER ET AL) 04 September 1990. See entire Abstract.	1-3

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers .. , because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-3, drawn to a method of determining the effectiveness of a cancer therapy.

II. Claim 4, drawn to a method for determining the rate of growth of a tumor.

III. Claims 5-16, drawn to a method to diagnose the presence or absence of the HER-2 protein.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-3

4. ☐ As all searchable claims could be searched without the payment of any additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest was made by the applicant with the payment of the additional search fees.